Functional Characterization of Paillotin: An Immune Peptide Regulated by the Imd Pathway with Pathogen-Specific Roles in *Drosophila* Immunity

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Abstract

Insects, such as *Drosophila* melanogaster, rely on innate immune defenses to combat microbial threats. Antimicrobial peptides (AMPs) play an important role in limiting pathogen entry and colonization. Despite intensive research into the regulation and biochemical properties of AMPs, their exact significance in vivo has remained uncertain due to the challenges of mutating small genes. Fortunately, recent technologies have enabled the mutation of individual AMP genes, overcome previous obstacles, and opened new avenues for research. In this study, we characterized one novel host-defense peptide, Paillotin (IM18, *CG33706*), using loss-of-function mutants. Paillotin is an ancient host defense peptide of Diptera, regulated by the Imd pathway. Loss of Paillotin does not impact the activity of either the Imd or Toll pathways. Importantly, we found that *Paillotin* mutants are viable but exhibit increased susceptibility to specific infections, particularly *Providencia burhodogranariea*. Paillotin was further found to contribute synergistically to defense against *P. burhodogranariea* when combined with other AMPs. However, we did not detect direct microbicidal activity of Paillotin *in vitro* in our hands. Taken together, our findings identify Paillotin as a novel host defense peptide acting downstream of Imd signaling, advancing our understanding of the *Drosophila* antimicrobial response.

Keywords: antimicrobial peptide, host–pathogen interactions, *Drosophila*, immunity

Introduction

Insects inhabit environments rich in microbes, necessitating a robust immune system to defend against opportunistic pathogens [1]. Unlike vertebrates, insects rely solely on innate immune defense mechanisms. The conservation of innate immunity across *Metazoans* and the powerful genetics of *Drosophila melanogaster* make this insect a powerful model for studying immune responses [2–4].

Flies exhibit behavioral immunity to prevent pathogens from entering their body cavity, such as grooming and avoidance of pathogens [5,6]. Once pathogens breach this barrier, immune responses are activated in different body regions to limit growth. Local immunity occurs at initial pathogen entry sites, like the gut or trachea. Systemic immunity involves cellular and humoral responses that take place in the body cavity upon systemic infection. Cellular responses involve hemocytes (blood cells), while humoral responses involve the production of host defense peptides as well as other effectors secreted by hemocytes and the fat body, an organ analogous to the mammalian liver [1,2]. Two NF-κB signaling pathways, the Toll and Imd pathways, regulate the humoral response. The principal effectors downstream of the Toll and Imd signaling pathways consist of AMPs, which are synthesized by both the fat body and hemocytes and then secreted into the hemolymph. AMPs are positively charged and can bind to the negatively charged membranes of microorganisms, disrupting membrane integrity through pore formation [1,7]. Upon infection, their expression is markedly increased to high levels in *Drosophila*, notably *Drosomycin* and *Diptericin,* which are often used as readouts for Toll and Imd activity, respectively [8]. Besides microbe killing, AMPs have been implicated in multiple processes such as tumor suppression [9,10], microbiota control [11], and neurodegeneration [12,13]. Currently, *D. melanogaster* is known to possess eight families of inducible AMPs, including antifungal peptides like Drosomycin, Baramicin, and Metchnikowin; Cecropins (four isoforms) exhibiting both antibacterial and antifungal activities *in vitro*; and Drosocin, Attacins (four isoforms), Defensin, and Diptericins (two isoforms), primarily displaying antibacterial activity [14–21]. Additionally, the *Drosophila* genome encodes numerous other host defense peptides, such as the Toll-regulated *Daisho* genes (two genes),

Bomanins (twelve genes), peptides encoded by *Baramicin A*, and the Imd-regulated peptide Buletin (product of the *Drosocin* gene), whose antimicrobial activity *in vitro* remains to be fully demonstrated, but functional studies have indicated their importance *in vivo* for surviving microbial infections [22–26].

Functional studies using single or combined mutations have demonstrated that these host defense peptides largely account for the critical roles of the Imd and Toll pathways in host survival following infection with Gram-negative bacteria, Gram-positive bacteria, or fungi. The antibacterial peptides Diptericin, Cecropin, Attacin, Drosocin, and Buletin account for most of the Imd pathway–mediated resistance to Gram-negative bacterial infection [25,27–29]. Drosomycin, Metchnikowin, Daisho (two genes), Baramicin A mediate much of the Toll-mediated resistance to fungal infection [23,24,26,27,30] , and Bomanins (twelve genes) appear to be the most important family of Toll-regulated host defense peptides contributing to resistance against Gram-positive bacteria and fungi [22,27,31]. These host defense peptides display both additive and synergistic effects, along with a high degree of specificity, whereby individual peptides greatly contribute to survival against a defined pathogen [27,29,32]. Some immune-induced peptides regulated by Toll and Imd have regulatory functions. This includes GNBP-like3 and Ibin A/B which have been reported to modulate Toll pathway activity, as well as Edin, which has been implicated in regulating hemocyte sessility [33–35].

Despite significant advances in understanding *Drosophila* host defense peptides, many peptides remain underexplored. The *Drosophila* genome encodes several other immune-induced peptides that remain poorly characterized, including notably Listericin, CG4269, and IM18 [1,36,37].

In this article, we have characterized the function of IM18, which we renamed Paillotin. We showed that Paillotin is an ancient host defense peptide regulated by the Imd pathway, which does not affect the regulation of the Imd or Toll pathways. Importantly, we found that *Paillotin* mutants exhibit increased susceptibility to specific infections, particularly *Providencia burhodogranariea*. Paillotin was also found to contribute synergistically to defense against *P. burhodogranariea* when combined with other AMPs, specifically Drosocin, Diptericins, and Attacins. However, we did not detect direct microbicidal activity of Paillotin *in vitro*. Taken together, our findings identify Paillotin as a novel host defense peptide

downstream of Imd signaling, advancing our understanding of the *Drosophila* antimicrobial response.

Results

Paillotin is a conserved host defense peptide in *Diptera*

A previous proteomic analysis identified an immune inducible peptide called Immune-induced Molecule 18 (IM18) based on its mass by MALDI-TOF [38]. We rename the *IM18* gene (*CG33706*) as *Paillotin* (*Pai*) from André Paillot, who in 1920 discovered the humoral immune response of insects [39,40]. The gene encoding Paillotin was identified as *CG33706* in a region annotated as polycistronic with another gene, *CG10332*, making it challenging to distinguish their expression patterns (Figure 1*a*); the *CG10332-RA* transcript is identical to the *Pai-RA* transcript and contains the full coding sequence of both *CG10332* and *Pai*. Indeed, in FlyAtlas data, *IM18* and *CG10332* are shown with identical expression levels [41]. However, the characterization of IM18 as an immune-induced peptide pointed to the existence of two distinct gene products, perhaps regulated by the distinct transcripts *Pai-RB* and *CG10332-RA.* To confirm this, we monitored the expression of either *CG10332-RA* alone or the cumulative products of *CG10332-RA* and *Pai-RB* upon septic injury with either the Gram-negative bacterium *Escherichia coli* (*E. coli*) or the Gram-positive bacterium *Micrococcus luteus* (*M. luteus*). The results showed that the mRNA encoding *Pai-RB* (Figure 2*a*, *b*) but not *CG10332-RA* was strongly induced upon septic injury (Figure 2*c*, *d*), confirming that only *Pai-RB* is immune-induced at the transcriptional level.

The *Paillotin* gene encodes a 71-residue precursor peptide composed of a signal peptide, dipeptidylpeptidase motif, and mature peptide (Figure 1*b*). To investigate the evolutionary conservation of Paillotin, we used a recursive tBLASTn approach to screen outgroup fly species [42]. Orthologues of Paillotin were identified in *Diptera* families such as *Tephritidae* and *Diopsidae*, which share a common ancestor with *D. melanogaster* approximately 126 million years ago [43] (Figure 1*b*). The C-terminal domain displayed the highest level of conservation across species, suggesting a critical role in Paillotin function. The presence of conserved features, such as the two-residue dipeptidyl peptidase cleavage site (XA/XP) and a positive

117 charge (+3.89 in *D. melanogaster*) commonly found in AMPs [24,44], supports the hypothesis
118 that Paillotin plays a key role in host defense and could have microbicidal activities. This
119 evolutionary conservation underscores Paillotin as an ancient host defense peptide in *Diptera*.
120

121 ***Paillotin* is regulated by the Imd pathway**

122 To explore the expression pattern and regulation of *Paillotin* during systemic immune
123 responses, we analyzed transcript levels in wild-type, Toll, and Imd pathway-deficient flies
124 (Figure 2). Specifically, we challenged w^{1118} (wild-type), spz^{rm7} (deficient for the Toll pathway),
125 $Relish^{E20}$ (deficient for Imd pathway) and $Relish^{E20}$, spz^{rm7} double mutant flies with either *E. coli,*
126 an inducer of the Imd pathway (Figure 2*a*), or *M. luteus,* an inducer of the Toll pathway (Figure
127 2*b*). Expression of *Paillotin* was monitored at 8h, 16h, and 24h post-infection using qRT-PCR.
128 We observed that *Paillotin* gene expression was strongly induced after both *M. luteus* and *E.*
129 *coli* infections in w^{1118} flies at all time points. However, this induction was completely abolished
130 in $Relish^{E20}$ and $Relish^{E20}$, spz^{rm7} double mutants, but not in spz^{rm7} flies, indicating *Paillotin* is
131 regulated by the Imd pathway (Figure 2*a*, *b*). Consistent with this expression profile, we found
132 multiple putative NF-κB responsive motifs within the first 200bp of promoter sequence
133 upstream of gene transcription start sites of *Paillotin* [24,45] including five motifs uniquely
134 matching the *Relish* consensus motif sequence (GGRDNNHHBS), one motif matching a
135 *Dif/Dorsal* (GGGHHNNDVH) binding site, and two κB motifs with putative matches to either
136 *Relish* and/or *Dif/Dorsal* binding sites. In contrast, no NF-κB binding motifs were identified in
137 the 250bp upstream region of *IM18-RA/CG10332-RA*, consistent with its non-immune
138 inducibility.

139 A recent proteomic analysis of *Drosophila* hemolymph following bacterial infection [46]
140 indicated that Paillotin peptide was strongly induced in the hemolymph of wild-type flies
141 infected with *M. luteus* or the Gram-negative bacterium *Pectobacterium carotovorum* (*Ecc15*),
142 and this induction was abolished in $Relish^{E20}$ flies (Figure 2*e*). Consistent with its regulation by
143 the Imd pathway, *Paillotin* was more induced upon infection with *Ecc15* than *M. luteus*. We
144 conclude that Paillotin is a novel host defense peptide regulated at the transcriptional level by
145 the Imd pathway and secreted into the hemolymph.
146

147 **Identification and characterization of *Paillotin* mutants**

148 The *Drosophila* genome nexus represents a global sampling of the genetic diversity of *D.*
149 *melanogaster* wild types subjected to genome sequencing [47]. Using a combination of the
150 genome nexus and more detailed variant information in the *Drosophila* Genetic Reference
151 Panel (DGRP) [48], we identified two strains from Kenya (KR7, KR4N) encoding a premature
152 stop codon in *Paillotin* ("Gly37∗", numbering per start codon). Globally, we could assess the
153 *Paillotin* region in 789 genome-sequenced strains, finding 39 that encode putative loss-of-
154 function mutations (Table S1). The majority of these mutant strains were collected from Winters
155 California and Raleigh North Carolina, which have a high frequency of putative loss-of-
156 function mutations (~26% and ~8% respectively within these populations). We characterized
157 two of the loss-of-function mutations segregating in the DGRP further (Figure 3*a*). The first
158 mutation (2R_19488505_DEL) is found in 17 genotypes from the DGRP, including DGRP-41,
159 which deletes 61 nucleotides within the Paillotin mature peptide coding region, leading to a
160 frameshift and protein truncation (referred to as $Pai^{\triangle 41}$). The second mutation
161 (2R_19488690_SNP), found in DGRP-370 ($Pai^{\triangle 370}$), involves a C-T transition that disrupts
162 the *Paillotin* start codon. We utilized these naturally-occurring mutations to study the role of
163 *Paillotin* in host defense by backcrossing $Pai^{\triangle 41}$ and $Pai^{\triangle 370}$ into the w^{1118} iso DrosDel
164 background for seven generations.

165 In addition, we used a third mutant referred to as $Pai^{\triangle SFH}$, a gift from the Guangzhou
166 Drosophila Resource Center (Figure 3*a*), which was generated by CRISPR-Cas9 and involves
167 a 20-nucleotide deletion that causes a complex frameshift, resulting in a nonsense peptide. The
168 precise nucleotide alterations associated with each *Paillotin* mutant allele used in this study are
169 presented in Supplementary Figure S1. For $Pai^{\triangle SFHI}$, only the *X* chromosome was replaced by
170 the w^{1118} iso DrosDel *X* chromosome.

171 RT-qPCR confirmed reduced *Paillotin* gene expression in $Pai^{\triangle 41}$ and $Pai^{\triangle 370}$, while
172 *Paillotin* expression was completely absent in $Pai^{\triangle SFHI}$ (Figure S2). We next performed a
173 MALDI-TOF analysis of hemolymph from adult flies infected with *P. c. carotovara Ecc15*. The
174 results revealed the absence of the 4625Da Paillotin peaks in $Pai^{\triangle 41}$, $Pai^{\triangle 370}$, and $Pai^{\triangle SFHI}$ flies
175 (Figure 3*b*). All the mutants were viable without morphological defects.

176

177 ***Paillotin* does not regulate the Imd and Toll pathways**

178 Some host defense peptides, such as mammalian LL37 or β-Defensins, have been shown
179 to function similarly to cytokines, modulating immune system activity [49,50]. This prompted
180 us to assess whether Paillotin could influence Toll and Imd pathway activity. To address this,
181 we monitored the expression of *Diptericin A* (a readout of the Imd pathway) in wild-type (w^{1118})
182 and *Paillotin* -deficient flies ($Pai^{\Delta 41}$ and $Pai^{\Delta 370}$) at 6- and 12-hours post-infection with *Ecc15*
183 and *Drosomycin* (a readout of the Toll pathway) at 8- and 24-hours post-infection with *M. luteus*
184 (Figure 4*a*, *b*). The expression levels of *Diptericin A* in $Pai^{\Delta 41}$ and $Pai^{\Delta 370}$ mutants were
185 comparable to those in wild-type flies at both time points, indicating no effect of *Paillotin* on
186 Imd pathway activity. Similarly, the expression of *Drosomycin* in $Pai^{\Delta 41}$ and $Pai^{\Delta 370}$ mutants
187 showed no significant difference compared to w^{1118}, suggesting that *Paillotin* is not involved in
188 regulating the Toll pathway. Together, these results demonstrate that *Paillotin* is not a regulator
189 of the Imd or Toll pathways.

190

191 ***Paillotin* mutant flies are susceptible to *P. burhodogranariea* infection**

192 Recent studies have demonstrated that the deletion of a single AMP gene can significantly
193 increase susceptibility to specific pathogens. For instance, the *Diptericin A* mutation leads to
194 marked susceptibility to *Providencia rettgeri*, while the *Diptericin B* mutation greatly reduces
195 resistance to *Acetobacter sp.* [29,32]. To investigate the role of Paillotin in resistance to
196 infections, we examined its contribution against a range of Gram-negative, Gram-positive
197 bacteria, and fungi. In these experiments, we compared the survival of $Pai^{\Delta 41}$, $Pai^{\Delta 370}$, and
198 $Pai^{\Delta SFHI}$ flies to that of the wild-type (w^{1118}), $Relish^{E20}$, $Bom^{\Delta 55}$, and spz^{rm7} immune-deficient
199 mutants. Flies were systemically infected by bacteria using a needle dipped in a bacterial pellet,
200 a mode of infection that causes cuticle injury. To exclude a role of *Paillotin* in resistance to
201 injury, we first monitored the susceptibility of *Paillotin* mutants to clean injury, and did not
202 detect any susceptibility after clean injury alone (Figure S3). Regarding Gram-negative bacteria,
203 there was no statistically significant difference in survival between wild-type and *Paillotin*
204 mutants following infections with *P. rettgeri*, *Ecc15*, or *Enterobacter cloacae* (Figure S4*a-c*).
205 However, all three *Paillotin* mutant flies ($Pai^{\Delta 41}$, $Pai^{\Delta 370}$, $Pai^{\Delta SFHI}$) exhibited a modest but
206 significant increase in susceptibility to septic injury with *Providencia burhodogranariea* (*P.*

207 *burhodogranariea*) (Figure 5*a*). This susceptibility was less marked than *Relish*^{E20} flies,
208 indicating that other factors downstream of the Imd pathway contribute to survival upon *P.*
209 *burhodogranariea* infection. In addition, we observed higher *P. burhodogranariea* bacterial
210 load in *Paillotin* mutants compared to wild-type, indicating that Paillotin suppresses bacterial
211 growth (Figure 5*b*). For Gram-positive bacteria, *Paillotin* mutants showed no consistent
212 increased susceptibility to *Enterococcus faecalis* (Figure 5*c*), *Bacillus subtilis*, *Listeria*
213 *monocytogenes*, or *Staphylococcus aureus* infections (Figure S4*d-f*). Similarly, fungal
214 infections with *Beauveria bassiana* (Figure 5*d*), *Metarhizium anisopliae*, or *Metarhizium*
215 *robertsii* did not reveal any increased susceptibility of *Paillotin* mutants compared to wild-type
216 (Figure S4*g-h*).
217

218 **No *in vitro* activity of Paillotin peptide**

219 Cecropins were the first inducible AMPs identified in moths [14]. *In vitro* studies have
220 shown that Cecropins are highly effective against a wide range of Gram-negative bacteria at
221 concentrations lower than those induced in insects during infection (25–50 μM) [51]. To
222 investigate the antimicrobial potential of Paillotin *in vitro*, we produced the peptide and
223 evaluated its activity against *P. burhodogranariea* at 100μM alone or in combination with
224 sublethal concentrations of Cecropin A. However, Paillotin exhibited no detectable
225 microbicidal activity against *P. burhodogranariea in vitro* (Figure S5*a*). It also failed to inhibit
226 the growth of other Gram-negative bacteria, including *Ecc15* and *E. coli* (Figure S5*b*, *c*). Thus,
227 we found no activity of Paillotin *in vitro* under these conditions.
228

229 **Paillotin synergistically contributes to the defense against *P. burhodogranariea***
230 **with other AMPs.**

231 Recent studies have demonstrated synergistic or additive cooperation among AMPs,
232 notably against *P. burhodogranaria*. While single mutations in *Drosocin*, *Attacins*, or
233 *Diptericins* have minimal overall effect, a compound mutant referred to as the "*Group B"* AMP
234 mutant lacking *Drosocin*, *Attacins A, B, C* and *D*, and *Diptericins A* and *B* [full genotype: w^{1118};
235 $AttC^{Mi}$, $Dro\text{-}AttAB^{SK2}$, Dpt^{SK1}; $AttD^{SK1}$], displays marked susceptibility to *P. burhodogranariea*
236 infection [27]. To investigate potential interactions between *Paillotin* and these AMPs, we used

genetic crosses to combine the *Paillotin* mutation with *Group B* mutants. We initially infected *Group B* mutants, *Paillotin* mutants, and *Group B, Paillotin* compound mutants [full genotype: w^{1118}; $AttC^{Mi}$, $Dro\text{-}AttAB^{SK2}$, Dpt^{SK1}, *Pai*] with *P. burhodogranariea*. However, we observed no synergistic or additive effects, as the introduction of *Paillotin* mutations into *Group B* mutants did not further increase susceptibility. This lack of an observable effect may be due to the already high susceptibility of *Group B* AMP mutants to this bacterium, even at a low inoculum (OD_{600}=0.1), potentially masking any additional contribution from the *Paillotin* mutation (Figure 6, dashed curves). To circumvent this, we assessed the impact of *Paillotin* homozygous deficiency in flies heterozygous for *Group B* AMP mutations (*Group B/+*), which depresses the inducibility of AMPs compared to homozygotes [27]. Notably, in this sensitized genetic background, the absence of *Paillotin*, regardless of the allele used, significantly increased susceptibility of *Group B/+* flies to *P. burhodogranariea* (Figure 6*a-c*, solid curves). This co-occurring loss of resistance reflects a synergistic effect on the Hazard Ratio (HR), evidenced by increased mortality beyond additive effects (*Paillotin* alone, HR =-0.17, p =0.01; *Group B* alone, HR =-1.06, p<0.001; *Paillotin*Group B* interaction, HR =-0.15, p=0.02). In addition, a marginally significant trend consistent with a synergistic effect between *Paillotin* and *Group B* mutants was also seen for infections with the Gram-negative bacteria *Providencia rustigianii* and *Providencia vermicola*, but not *E. coli* (Figure S6). This supports the notion that Paillotin confers a fairly specific resistance to *P. burhodogranariea*, perhaps also extending to other *Providencia* species.

Overexpression of Paillotin restores resistance in Paillotin-deficient flies against *P. burhodogranariea*

The observation that three distinct null mutations in *Paillotin* are associated with a higher susceptibility and the synergistic effect between Paillotin and other AMPs against infection provide strong evidence for a role of this host defense peptide in resisting *P. burhodogranariea*. To reinforce this finding, we performed a genetic rescue experiment by combining the *Paillotin* mutation with a *UAS-Pai* transgene. We initially observed that overexpression of *Paillotin* in a wild-type background was not sufficient to enhance resistance to *P. burhodogranariea* (Figure S7). In contrast, overexpression of *Paillotin* using either the *C564-Gal4* (a Gal4 driver with

strong expression in the fat body) or *Actin-Gal4* (ubiquitous) driver restored pathogen resistance to near wild-type levels in the *Pai*$^{\triangle 41}$ (Figure 7*a*) and *Pai*$^{\triangle 370}$ (Figure 7*b*) mutant backgrounds. The observation that three distinct *Pai* alleles confer susceptibility, together with the results of the rescue experiment, collectively demonstrates that Paillotin acts as a pathogen-specific host defense peptide essential for restricting *P. burhodogranariea* proliferation.

The Imd and Toll signaling pathways are essential for insect immunity, with double pathway-deficient flies exhibiting extreme susceptibility to infection due to their inability to upregulate hundreds of immune effector genes, particularly AMPs [52]. Previous work has demonstrated that transgenic overexpression of individual AMPs in some cases is sufficient to restore resistance in *Imd, Toll-deficient* flies [53]. Building on this paradigm, we generated flies overexpressing *Paillotin* using *Actin-Gal4* or *C564-Gal4* drivers in *Relish*^{E20} mutant flies (Figure 7*c*). However, *Paillotin* overexpression failed to confer protection against *P. burhodogranariea* infection. While we cannot exclude that Paillotin was not expressed at a sufficient level to overcome the extreme susceptibility of *Relish* mutants, this suggests that *Paillotin* alone is insufficient and the host requires the presence of other AMPs for the defense response to function effectively, consistent with the synergistic interactions observed with *Group B*/+ heterozygous mutants.

Discussion

In this study, we characterized Paillotin, an evolutionarily conserved host-defense peptide regulated by the Imd pathway. Use of three *Paillotin* mutants reveals a role of *Paillotin* in the resistance to *P. burhodogranariea* infection, and perhaps other moderately virulent *Providencia* species, without altering survival to other tested bacterial or fungal species. Furthermore, the *Paillotin* mutation enhances the susceptibility to *P. burhodogranariea* in flies heterozygous for mutations in *Drosocin*, *Diptericins A and B*, and *Attacins A, B, and C.* Taken together, the consistent susceptibility of three independent *Paillotin* alleles to *P. burhodogranariea*, accompanied by elevated bacterial loads, the resistance of *Paillotin*-deficient flies to other tested pathogens, and the successful rescue of the phenotype by overexpression, all provide strong evidence that *Paillotin* functions as a host defense peptide specifically effective against

295 this bacterium. A high degree of specificity has been observed for other host defense peptides,
296 whose inactivation frequently causes pathogen-specific immune deficiency. This has been
297 illustrated not only for Diptericin A and B as described previously [29,32], but also for Drosocin,
298 which provides significant protection against *E. cloacae* [25,27]. The existence of such
299 specificity between a defined host defense peptide and particular pathogens suggests that these
300 genes have been evolutionarily maintained under selective pressure imposed by specific
301 microbes encountered in natural environments [54].

302 Previous studies have shown that resistance to *P. burhodogranariea* involves multiple
303 antimicrobial peptides, including Buletin (encoded by the *Drosocin* gene), Diptericins A and B,
304 and Attacins A, B, C, and D. While single AMP mutations in many genes do not greatly impact
305 survival following infection with *P. burhodogranariea*, compound mutants lacking *Drosocin*,
306 *Diptericins* and *Attacins* exhibit increased susceptibility, suggesting the presence of a
307 synergistic effect among these peptides [27]. More recently, the absence of Buletin—a peptide
308 generated by furin cleavage of the Drosocin precursor—results in notable survival differences
309 following *P. burhodogranariea* infection [25]. Thus, host resistance to *P. burhodogranariea* is
310 mediated by the collective action of multiple host defense peptides, and our study now adds
311 Paillotin to this list. Future studies may further reveal additional roles of Paillotin, either alone
312 or in combination with other host defense peptides, in resistance to other pathogens.

313 We were unable to detect any direct *in vitro* microbicidal activity of Paillotin against *P.*
314 *burhodogranariea* and other bacteria, either alone or in combination with Cecropin. This
315 reflects a common mismatch between in vitro and in vivo activities. Despite demonstrating
316 effective antimicrobial activity in vivo in relevant animal models, many AMPs show minimal
317 or no activity in standard minimal inhibitory concentration (MIC) or minimal microbicidal
318 concentration (MMC) assays when tested under physiological salt concentrations or in serum-
319 containing environments [55–58]. Several factors could explain this lack of detectable activity:
320 i) Paillotin may depend on host-specific factors for its function, such as the requirement for a
321 cofactor or modulation by environmental cues (e.g., pH or ionic strength), or ii) synergistic
322 interactions with other immune molecules that are only present *in vivo*. Similar synergistic
323 mechanisms have been observed in other systems. For instance, in *Galleria mellonella* (the

324 greater wax moth), an anionic antimicrobial peptide (anionic peptide 2) enhances lysozyme-
325 mediated membrane perforation, facilitating the elimination of *E. coli* [59]. Furthermore, AMPs
326 often enhance each other's efficacy through complementary mechanisms of action [21,60]. For
327 instance, the bumblebee linear peptides hymenoptaecin and abaecin exhibit a synergistic
328 interaction against *E. coli*. Although abaecin alone shows no detectable activity at
329 concentrations up to 200 μM, hymenoptaecin disrupts the bacterial membrane, allowing
330 abaecin to enhance bactericidal effects at doses as low as 1.25 μM [61]. In *Tenebrio molitor*,
331 RNAi-mediated knock-down of the defensin Tenecin-1 did not affect host survival but reduced
332 bacterial load following *S. aureus* infection. However, simultaneous knock-down of Tenecin-1
333 with other AMPs, such as the coleoptericin Tenecin-2 or the attacin Tenecin-4, resulted in
334 increased mortality and higher bacterial loads, highlighting functional complementarity among
335 AMPs during infection [62]. In vitro experiments showed that Attacin, an antibacterial protein
336 that accumulates in the hemolymph of the giant silk moth *Hyalophora cecropia*, compromises
337 the permeability barrier function of the *E. coli* outer membrane. Pretreatment with Attacin
338 significantly increases the sensitivity of *E. coli* to Cecropin B, another antimicrobial protein
339 present in the hemolymph of *H. cecropia* [63]. In addition, recent studies have pointed to the
340 role of host defense peptides in resilience mechanisms, including tolerance to host immune
341 molecules [46] and pathogen toxins [26,31]. However, we did not detect a role for Paillotin in
342 resilience to the fungal toxins Destruxin A or Verruculogen (Figure S8). Interestingly, in
343 contrast to recent studies [26,31], we did not detect a susceptibility of Toll-deficient spz^{rm7} flies
344 to these two toxins (Figure S8). Of note, the description of DIMs has been done largely by
345 collecting peptides in Trifluoroacetic acid, which is known to modify peptides by, for example,
346 deamidating NG motifs [24,64]. It is possible that the endogenous peptide products of the host
347 immune response differ from the peptides observed in proteomic analysis and synthetic
348 peptides used in recent studies, in unknown ways. Early studies on AMPs typically found
349 striking activity *in vitro* using peptide extracts purified from endogenous hemolymph or cell
350 line supernatant [14,17,19,21,65]. Future studies may benefit from a return to such approaches,
351 including gel overlay experiments that can simplify the isolation of the activity of single
352 peptides from endogenous samples [66] or collecting crude peptide extracts from
353 overexpressing cells or larval hemolymph. However, future studies might also explore whether

354 Paillotin protects against virulence factors specific to *P. burhodogranariea*. For now, despite
355 displaying many features of AMPs, we cannot conclusively classify Paillotin as a bona fide
356 AMP. It should be classified as a host defense peptide until its molecular mode of action is fully
357 determined.

358 The use of compound module-deficient mutants has revealed the major role of the Imd
359 pathway, and to a lesser extent, melanization, but not the cellular response to survive *P.*
360 *burhodogranariea* infection [67]. Since Paillotin does not affect Toll and Imd pathway activities,
361 we cannot exclude a role of this peptide in the melanization reaction. However, this role would
362 have to be subtle as *Paillotin* mutants display a wild-type blackening reaction upon clean injury
363 (Figure S9*a*). Previous studies have shown that flies lacking 14 AMPs exhibit increased
364 microbiota abundance and altered composition in old flies, suggesting that AMPs are involved
365 not only in pathogen resistance but also in shaping the environmental microbiota [11]. However,
366 we did not detect any significant differences in gut microbiota load between wild-type flies and
367 *Paillotin* mutants (Figure S9*b*).

368 Interestingly, there is a relatively high frequency of loss-of-function mutations segregating
369 in wild *Drosophila* populations (Table S1), suggesting Paillotin is not integral to the generic
370 host defense. This observation is consistent with our results showing that the *Paillotin* mutation
371 affects defense against only a few bacteria out of the pathogens tested, suggesting it is
372 dispensable in defense against many microbes, but useful against some microbes like
373 *Providencia sp.* whose presence is stochastic in wild-caught flies [68].

374 To conclude, our study characterizes a new *Drosophila* host defense peptide, Paillotin,
375 which plays a specific role against *P. burhodogranariea*. IM18/Paillotin is the last gene
376 encoding an immune molecule identified by MALDI-TOF in the 1998 study [38] to be
377 characterized, following the successful characterization of Bomanins [30], Daisho [23], Buletin
378 [25], and Baramicin [24,26]. Together, these studies and the present article provide important
379 insights into the *Drosophila* humoral immune response, revealing a more complex picture than
380 initially anticipated. Using the power of *Drosophila* genetics to dissect how immune effectors
381 individually or collectively contribute to host defense provides a general principle on the
382 architecture of innate immune systems.

383 # Materials and Methods

384 ## Sequence Comparisons

385 Genomic sequences were obtained from GenBank default reference assemblies [69], from
386 the DPGP via the PopFly website (www.popfly.uab.cat)[47], and DGRP sequence data from
387 the DGRP database [48] (http://dgrp2.gnets.ncsu.edu/). Sequence comparisons and alignment
388 figures were prepared using Geneious R10 [70]. Putative NF-κB binding sites were annotated
389 using the *Relish* motif "GGRDNNHHBS" as described in Copley et al [45], along with a
390 manually curated amalgam motif "GGGHHNNDVH" described in Hanson et al [24].

391 Paillotin annotation across species was performed as described in Hanson et al [42]. In
392 brief, a recursive tBLASTn approach was used with an artificially high E-value (E < 1000) to
393 compensate for the short length of the AMP query sequence. All the best BLAST hits were
394 assessed manually. If no Paillotin was found in a genome or lineage, only the conserved C-
395 terminus was used as a query in a repeat attempt (improving sensitivity). No *Paillotin*
396 candidates were discovered outside *Brachycera*, which included screens of the sequenced
397 nematoceran genomes of various mosquitoes, *Chironomus riparius, Clogmia albipunctata,*
398 *Tipula unca, Lutzomyia longipalpis, Phlebotomus papatasi, Sylvicola cinctus,* and *Bibio marci*.

399 ## Fly Strains

400 Flies were maintained at 25°C on standard cornmeal-agar media. w^{1118} flies were used as
401 wild-type controls. The positive controls for infection assays for Gram-positive and Gram-
402 negative infections were spz^{rm7} and $Relish^{E20}$. The $Pai^{\Delta 41}$ and $Pai^{\Delta 370}$ mutations are loss-of-
403 function variants in the *IM18* gene within the DGRP, and $Pai^{\Delta SFHI}$ was generated via
404 CRISPR/Cas9. The guide RNAs that targeted *Pai* were cloned into the pUAST-attB plasmids
405 before injecting into the *y [1] M {RFP [3xP3.PB] GFP[E.3xP3] =vas-int.Dm}ZH-2A w [*];*
406 *M{3xP3-RFP.attP} ZH-86Fb* (BL24749) to get gRNA transgenic flies. The gRNA flies were
407 then crossed with flies containing nos-Cas9 to get mutants. The *UAS-Pai* constructs were
408 prepared using the TOPO pENTR entry vector and cloned into the pTW system. Genetic
409 variants were backcrossed into the DrosDel isogenic background over seven generations as
410 previously described [71] using the following primers that are specific to an indel in the *Pai*

411 locus common to both the *Pai*$^{\Delta 41}$ and *Pai*$^{\Delta 370}$, but which is absent in the DrosDel background:
412 Pai_41F (ACGTACGGTCAAAAGCAACTACTTCG) and Pai_R3mut1
413 (CCAATCCCAAGGGCACTCGTATATG), amplified with a 66°C annealing temperature. All
414 the fly strains used in the paper are listed in Table S2.

415 **MALDI-TOF Proteomic Analysis**

416 Hemolymph samples were collected from *Ecc15*-infected flies (OD600 = 200, 24 hours
417 post-infection, 50 flies/sample) using a nanoinjector (Nanoject, Drummond Scientific,
418 Broomall, PA) for MALDI-TOF analysis. Trifluoroacetic acid (0.1% TFA) was added to the
419 hemolymph before mixing with the acetonitrile matrix. Samples are processed by SPE (Zip-Tip
420 C18) before MALDI. Peaks were identified based on corresponding m/z values from previous
421 studies [24,72] in ISIC's Mass Spectrometry facility of EPFL. Spectra were visualized using
422 Origin software, with figures prepared in Prism 10.

423 **Gene Expression Analysis**

424 Total RNA was extracted from pooled samples of ten flies using TRIzol. cDNA was
425 synthesized using Takara Reverse Transcriptase. Quantitative PCR (qPCR) was performed
426 using PowerUP SYBR Green Master Mix with the primers. Results represent the average of
427 three independent experiments. Primers used in this study were:

428 *Drosomycin*-F 5'- CGTGAGAACCTTTTCCAATATGAT-3'

429 *Drosomycin*-R 5'- TCCCAGGACCACCAGCAT-3'

430 *Diptericin A*-F: 5'-GCTGCGCAATCGCTTCTACT-3'

431 *Diptericin A*-R:5'-TGGTGGAGTGGGCTTCATG-3'

432 *Rpl32*-F: 5'-GACGCTTCAAGGGACAGTATCTG-3'

433 *Rpl32*-R: 5'-AAACGCGGTTCTGCATGAG-3'

434 *Pai*-F: 5'-GATCCGTTTCCTGGTCCAGTGTG-3'

435 *Pai*-R: 5'-CCATGAAGCTGATCGCATTGTGC-3'

436 *CG10332*-F: 5'-TCAGAGGCTGCACCATCAGAAGT-3'

437 *CG10332*-R: 5'-CCGCTTCAGAGGCTGCACC-3'

438 **Microbial Culturing Conditions**

439 *Micrococcus luteus*, *Erwinia carotovora carotovora (Ecc15)*, and *Providencia*
440 *burhodogranariea* strain B were cultured overnight in LB broth at 29°C. *Enterobacter cloacae*,
441 *Escherichia coli* strain 1106, *Providencia rustigianii*, *Providencia vermicola, Providencia*
442 *rettgeri,* and *Staphylococcus aureus* were cultured overnight in LB at 37°C. *Enterococcus*
443 *faecalis*, *Bacillus subtilis,* and *Listeria monocytogenes* were cultured overnight in BHI at 37°C.
444 *Metarhizium anisopliae* and *Metarhizium robertisii* were cultured on malt agar plates at 25°C
445 for around two weeks until sporulation was observed. *Beauveria bassiana* strain R444
446 commercial spores were produced by Andermatt Biocontrol, products: BB-PROTEC. Bacteria
447 were pelleted at 4000 rpm for 15 minutes at 4°C, resuspended in PBS, and diluted to the desired
448 OD600 value indicated in the figure legends. The fungal material was filtered through glass
449 wool using 0.05% Tween-80 to collect spores and diluted to the desired concentration (in
450 spores/ml). All microbial strains used in this study are listed in Table S3.

451 **Microbiota Load Analysis**

452 Flies were collected 30 hours post-infection and surface-sterilized with 70% ethanol.
453 Whole-fly homogenization was performed in LB broth using a bead beater at 7200 rpm for 30
454 seconds. Serial dilutions of the homogenates were plated (100 μL per plate) on LB agar and
455 incubated at 29 °C for 24 hours. Colony-forming units (CFUs) were then manually counted.

456 For gut microbiota load analysis, 18-day-old conventionally reared flies were collected
457 and processed using the same procedure, except that MRS medium was used for culturing.
458 Plates were incubated at 29 °C for 48 hours prior to CFU quantification.

459 ***In Vitro* Antibacterial Activity**

460 The Paillotin peptide (45 residues) was synthesized by GenicBio with >95% purity, and
461 Cecropin A (from silk moth) was obtained from Sigma-Aldrich (≥97% purity). Peptides were
462 dissolved in water. Bacteria were cultured overnight and diluted to OD600 = 0.005 in LB broth,

463 then incubated for 2 hours. Paillotin peptide and Cecropin A were diluted in LB broth to the
464 desired concentration. Bacterial cultures (1 μL) were added to peptide solutions in a 96-well
465 plate, and OD600 was recorded every 10 minutes using a TECAN plate reader.

466 **Survival Assays**

467 Male flies (3-5 days old) were used for survival assays. The thorax of each fly was pricked
468 with a needle (diameter ~0.2mm) dipped in a bacterial suspension. For the fungi infection, flies
469 were rolled in the plates of *A. fumigatus* or the commercial spores of *B. bassiana,* or were
470 dipped into a spore solution of *M. anisopliae* or *M. robertisii*. Mortality was recorded daily.
471 Infected flies were maintained at 29°C or 25°C and transferred to fresh vials three times per
472 week.

473 **Toxin Injection**

474 Destruxin A (Sigma) was dissolved in high-purity dimethyl sulfoxide (DMSO) and
475 subsequently diluted with PBS to a final concentration of 8 mM. A volume of 9.2 nL of this
476 solution was administered to flies using the Nanoject III microinjector (Drummond) [26].
477 Verruculogen (Abcam) was prepared in DMSO at a concentration of 1 mg/mL, and 4.6 nL of
478 this solution was injected into the flies [31].

479 **Wounding experiment**

480 Clean injury was performed with a needle sterilized (diameter ~0.1mm) with an EtOH and
481 PBS wash. Cuticle blackening was categorized into normal, weak, or no melanization 24h post-
482 pricking at the injury site.

483

484 **Ethics.** This work did not require ethical approval from a human subject or animal welfare
485 committee.

486 **Data accessibility.** All data and code used in this study are publicly available at Dryad [73].

487 **Declaration of AI use.** We have not used AI-assisted technologies in creating this article.

488 **Authors' contributions.** Conceptualization: YT, XJY, MAH, BL. Data curation: YT, XJY,

MAH. Funding acquisition: BL, MAH. Investigation: YT, XJY, RJJ, FS. Methodology: YT, XJY, RJJ, MAH. Project administration: BL. Validation: YT, MAH. Resources: YT, XJY, MAH. Supervision: MAH, BL. Writing – original draft: YT, BL. Writing – review & editing: MAH, BL.

Conflict of interest declaration. We declare we have no competing interests.

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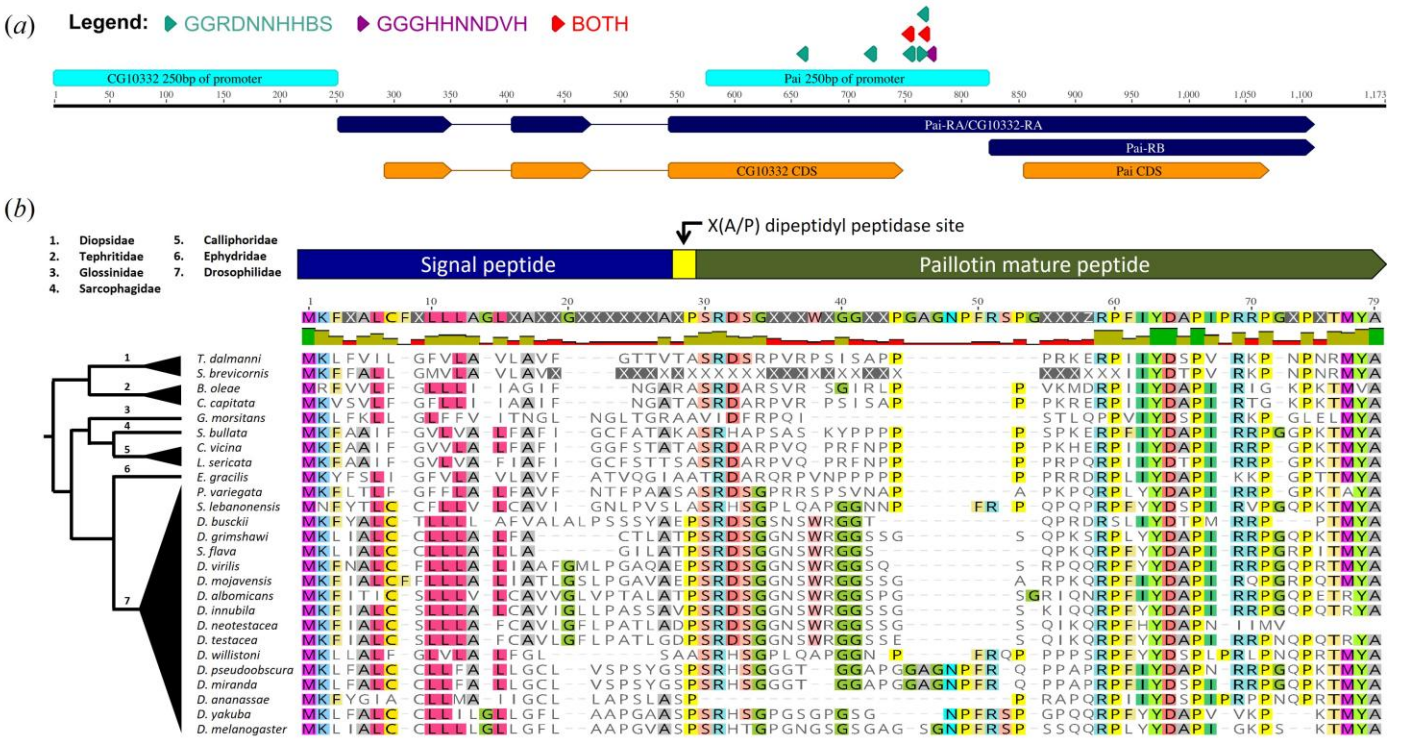
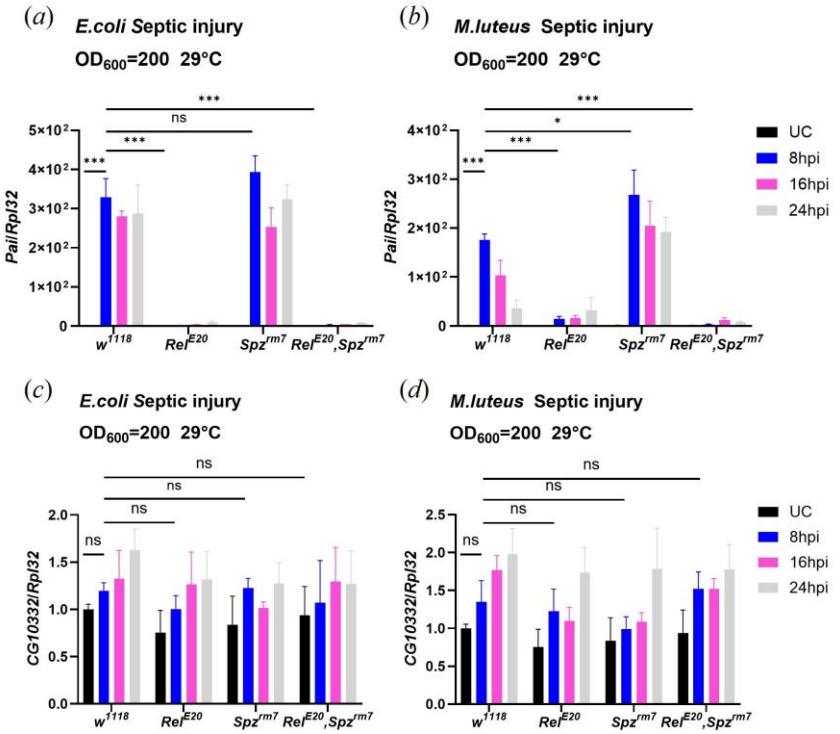


Figure 1: The *Paillotin* gene structure and conservation. (*a*) Schematic representation of the *Paillotin* (*Pai*) locus. The *Pai-RB* transcript is annotated as polycistronic, sharing a promoter and transcript with *CG10332* (*CG10332-RA*), indicating both gene CDS regions are co-transcribed. Promoter regions (250 bp upstream) for *Pai-RB* and *CG10332-RA* are shown in cyan. Colored triangles indicate putative NF-κB binding motifs: *Relish*-responsive motifs (GGRDNNHHBS) in teal, *Dif/Dorsal*-responsive motifs (GGGHNNNDVH) in purple. Red triangles represent sites containing overlapping matches to both motifs. (*b*) Sequence alignment of Paillotin orthologues across diverse *Brachyceran* flies revealed a conserved protein structure, including a signal peptide, a dipeptidyl peptidase cleavage site (XA/XP), and the mature Paillotin peptide. Highlighting corresponds to residues with >50% identity across the alignment.



(e)

Genotype	hpi	Pai peptide abundance (Relative to unchanged)	
		Ecc15	*M. luteus*
w^{1118}	2h	20.82	2.39
	6h	52.35	50.56
	24h	744.43	53.08
Rel^{E20}	6h	0.76	-
spz^{rm7}	6h	-	98.36

Figure 2: *Paillotin* is regulated by the Imd pathway. Transcript levels of *Paillotin*/*CG10332* in w^{1118}, $Relish^{E20}$, spz^{rm7}, and $Relish^{E20}$, spz^{rm7} flies collected at 8 h, 16h, and 24h post-*E. coli* infection (*a*, *c*) and *M. luteus* infection (*b*, *d*). (*e*) Quantified Paillotin peptide levels of w^{1118}, $Relish^{E20}$, and spz^{rm7} hemolymph upon either *Ecc15* or *M. luteus* infection (data extracted from (Rommelaere *et al.*, 2025)). Gene Expression was measured by qRT-PCR and normalized with w^{1118} UC (unchallenged) flies set as a value of 1. The data shown in the figure are based on three independent experiments, with at least 30 flies per genotype at each time point in each experiment. Error bars represent SEM. Statistical significance was determined using two-way ANOVA (*p<0.05, ** p<0.01, *** p<0.001, n.s. = not significant, p>0.05).

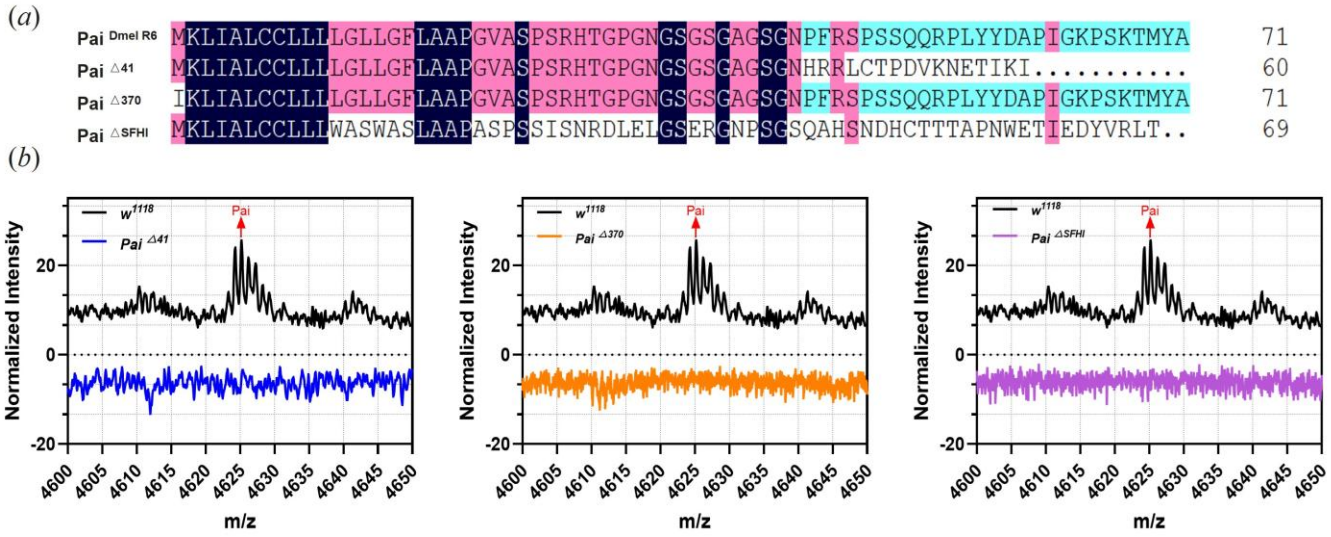


Figure 3: Description and validation of *Pai*$^{\triangle 41}$, *Pai*$^{\triangle 370}$, and *Pai*$^{\triangle SFHI}$ mutations. (*a*) Multiple sequence alignment between the Dmel_R6 reference genome Paillotin and Paillotin peptide variants. Conserved residues are highlighted by color coding according to sequence similarity. (*b*) MALDI-TOF proteomic analysis of hemolymph from *w*^{1118}, *Pai*$^{\triangle 41}$, *Pai*$^{\triangle 370}$, and *Pai*$^{\triangle SFHI}$ male flies collected 24 hours after septic injury with *Ecc15*.

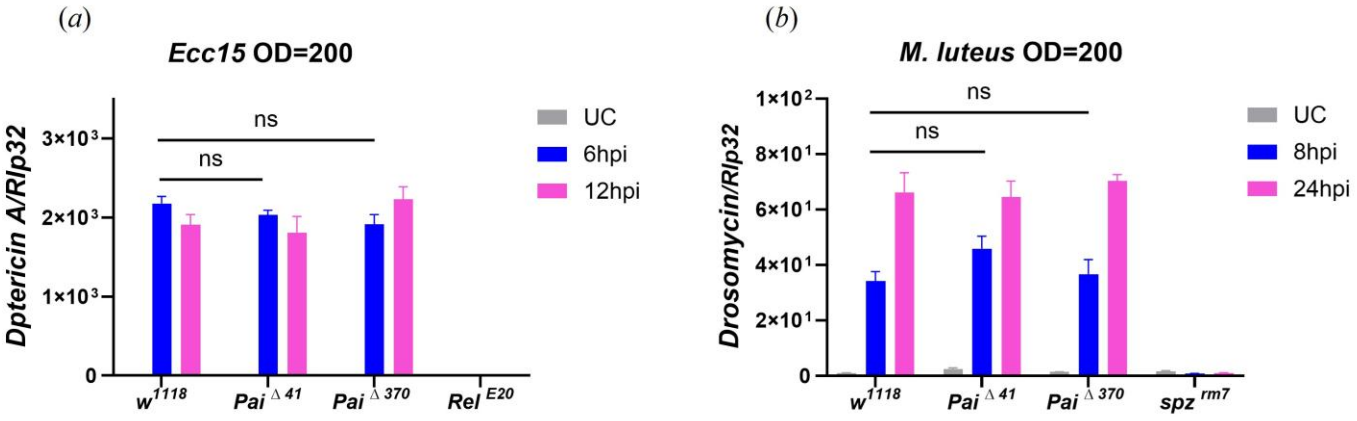


Figure 4: Paillotin does not impact the Imd or Toll pathways. The expression of *Diptericin A* (*a*) at 6 or 12h post-infection with *Ecc15* and *Drosomycin* (*b*) at 8h or 24h post-infection with *M. luteus* were monitored in w^{1118}, $Pai^{\Delta 41}$, and $Pai^{\Delta 370}$ flies. $Relish^{E20}$ and spz^{rm7} were used as the positive control. Expression was normalized to w^{1118} UC (unchallenged) flies set as a value of 1. The data shown in the figure are based on three independent experiments, with at least 30 flies per genotype at each time point in each experiment. Error bars represent SEM. Statistical analysis was performed using two-way ANOVA. For *Diptericin A*, significant effects were detected for genotype ($F(3,24) = 148.1$, $p < 0.0001$), time ($F(2,24) = 332.8$, $p < 0.0001$), and their interaction ($F(6,24) = 38.98$, $p < 0.0001$). For *Drosomycin*, significant effects were also observed for genotype ($F(3,24) = 72.82$, $p < 0.0001$), time ($F(2,24) = 191.5$, $p < 0.0001$), and interaction ($F(6,24) = 22.84$, $p < 0.0001$). However, Tukey's post hoc comparisons revealed no significant difference between w^{1118} and *Paillotin* mutants at any time point (n.s. = not significant).

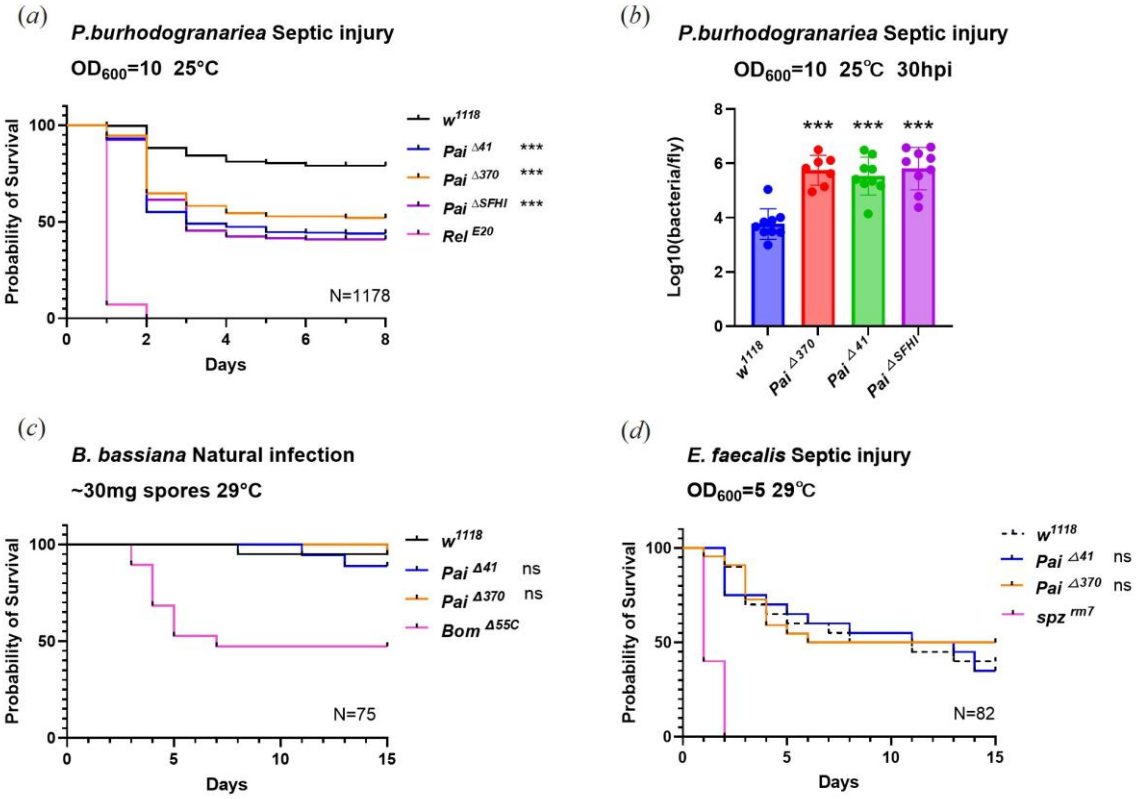


Figure 5: *Paillotin* mutants are susceptible to *P. burhodogranariea* infection. (*a*) The three *Paillotin* mutants exhibit increased susceptibility following septic injury with *P. burhodogranariea* at the indicated bacterial inoculum. (*b*) Increased bacterial load in *Paillotin* mutants at 30 hpi. Bacterial load was $\log_{10}$-transformed prior to statistical analysis. One-way ANOVA revealed a significant genotype effect ($F(3,30) = 18.95$, $p < 0.0001$). Post hoc Dunnett's test confirmed that all three mutant lines carried significantly higher bacterial loads than w^{1118} ($p < 0.0001$ for all comparisons). Data points represent the mean bacterial load per fly, derived from individual experiments using pools of four flies per genotype following *P. burhodogranariea* infection. No consistent effect of the *Paillotin* mutation on resistance to the Gram-positive bacterium *Enterococcus faecalis* (*c*) or the fungus *Beauveria bassiana* (*d*). Survival data were analyzed using the Kaplan–Meier method. Statistical significance is indicated relative to the w^{1118} (*** $p<0.001$, n.s. = not significant, $p>0.05$). N=total number of flies in experiments.

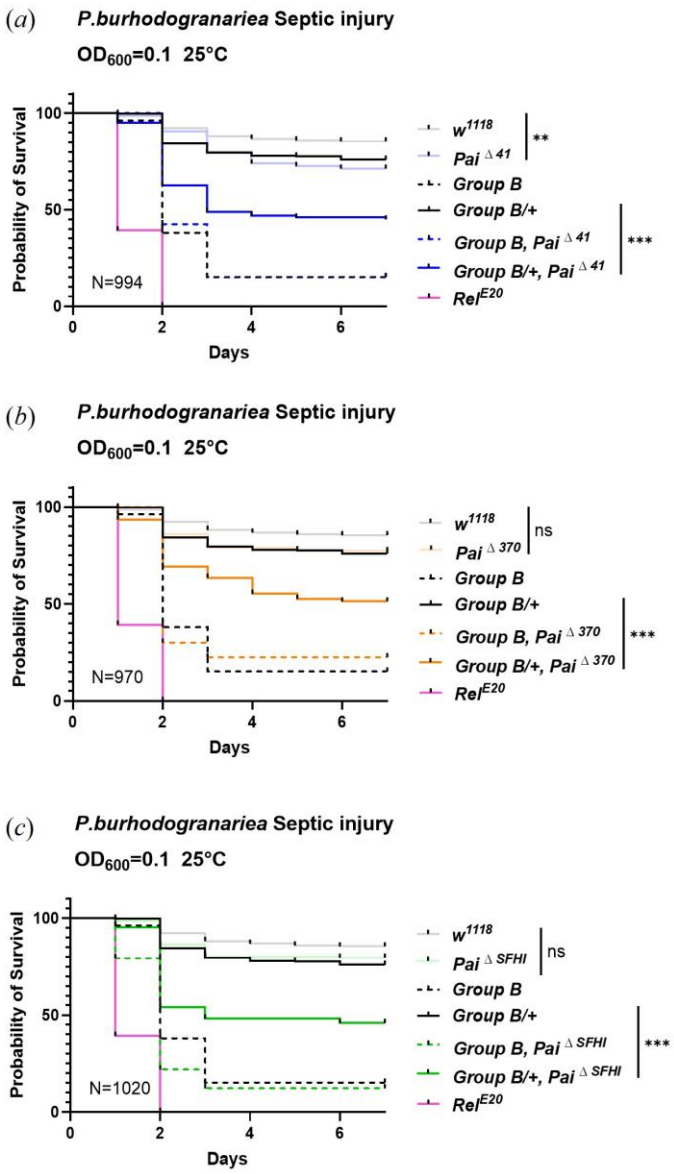


Figure 6: Paillotin synergistically contributes to defense against *P. burhodogranariea* with other Imd-regulated AMPs. (*a*-*c*) Survival curves showing the synergistic effects between different *Paillotin* mutants ($Pai^{\Delta 41}$, $Pai^{\Delta 370}$, $Pai^{\Delta SFHI}$) and *Group B* mutations (*Drosocin*, *Attacin A, B, C,* and *Diptericin A, B*) in the heterozygous background (*Group B/+*). Survival curves were analyzed using the Cox proportional hazards (CoxPH) model in R version 3.6.3. (** $p<0.01$, *** $p<0.001$, n.s. = not significant, $p>0.05$). N=total number of flies in experiments.

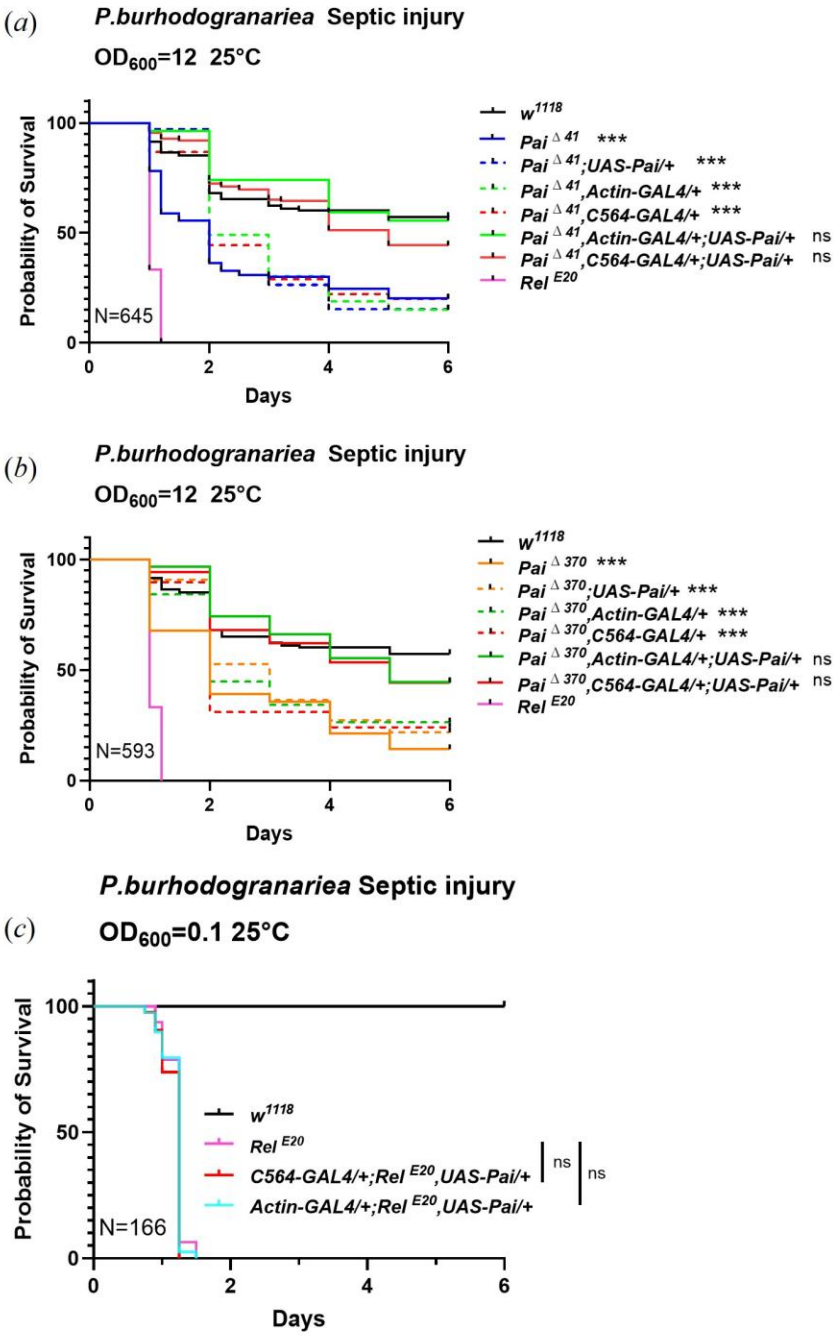


Figure 7: Over-expression of *Paillotin* rescues the resistance of *Paillotin*-deficient flies against *P. burhodogranariea*. (*a*-*b*) Overexpression of *Paillotin* via a combination of the *C564-Gal4* or *Actin-Gal4* and *UAS-Pai* constructs enhances the resistance of the Paillotin mutant to *P. burhodogranariea* infection. (*c*) Overexpression of *Paillotin* in the *Relish*^{E20} background failed to restore resistance. Survival curves were analyzed using the Kaplan-Meier method. (*** $p<0.001$, n.s. = not significant, $p>0.05$). N=total number of flies in experiments.

Supplementary material for

Functional Characterization of Paillotin: An Immune Peptide Regulated by the Imd Pathway with Pathogen-Specific Roles in *Drosophila* Immunity

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Table S1: Collectively, 39 of 789 genome-sequenced fly strains, including 18 of the 205 *Drosophila* Genetic Reference Panel lines encode a loss-of-function mutation in *Paillotin*. Lines encoding *Pai* putative loss-of-function mutations are annotated, alongside the genetic description of each allele. $Pai^{\Delta 41}$ "uncertain" and "alternate" alleles per the DGRP variant call dataset are both represented by a string of ~61 uncertain nucleotide calls (Ns) matching the pattern of allele 2R_19488505_DEL in the DPGP3 dataset (downloaded from PopFly). This suggests DGRP strains with "uncertain" allele annotations at base Dmel.R5_2R_19488505 also bear the 2R_19488505_DEL indel. Bolstered by this finding, we scanned the *Drosophila* genome nexus for strings of uncertain variant calls (Ns) that affect at least 3 contiguous codons, treating these as putative indels. This may yield an undercount by ignoring smaller indels, or an overcount by treating regions bearing multiple SNPs in linkage as indels. Nevertheless, this annotation effort draws attention to strains bearing potential loss-of-function mutations in *Paillotin* that may be relevant to future studies. DPGP indel strain summaries are grouped by the location of the first disrupted codon (within the signal or mature peptide).

Mutation	Allele	Reference allele	Alternate allele	Effect	Present in
Pai$^{\Delta 41}$	*2R_19488505_DEL*	(C)CAAT CGGAG CGTCG TAGTA CAGTG GTCGT TGCTG TGAGC TTGGA GACCT GAACG GATTT CC	(C)-	61bp deletion	**DGRP:** 28, 31, 41, 176, 227, 321, 362, 390, 406, 409, 491, 707, 843, 852, 879, 892, 911 **DPGP:** CHB381
Pai$^{\Delta 370}$	*2R_19488690_SNP*	C	T	loss of start codon	**DGRP:** 370 **DPGP:** USW_59 possibly heterozygous
Pai^{G37*}	*Pai^{G37*}*	GGA codon	TGA codon	codon 37 premature stop	**DPGP:** KR7, KR4N
Pai$^{\Delta M1}$	*Pai$^{\Delta M1}$*	ATG codon	ANG codon	putative start codon disruption	**DPGP:** USI06
Signal peptide + DPase indel	*multiple*	ATGAA GCTGA TCGCA TTGTG CTGCC TGCTC CTTTT GGGCC TCCTG GGCTT CCTAG CTGCT CCCGG CGTCG CCTCG CCA	multiple	indel beginning within signal peptide	**DGRP:** 385, 887 **DPGP:** NTH_07, NTH_15, USW_26, USW_37, USW_62, USW_68, USW_49, USW_76, AUS_04, EA_70N, MW_6-2
Mature peptide indel not including Pai$^{\Delta 41}$	*multiple*	TCTCG CCACA CTGGA CCAGG AAACG GATCG GGATC TGGAG CTGGG TCCGG AAATC CGTTC AGGTC TCCAA GCTCA CAGCA ACGAC CACTG TACTA CGACG CTCCG ATTGG GAAAC CATCG AAGAC TATGT ACGCC TGA	multiple	indel beginning within mature peptide not including *Pai$^{\Delta 41}$*	**DPGP:** USW_74, USW_87, FR_2N

```
Pai Dmel R6  ATCAAGCTGATCGCATTGTGCTGCCTGCTCCTTTTGGGCCTCCTGGGCTTCCTAGCTGCTCCCGGCGTCGCC   72
Pai Δ41      ATCAAGCTGATCGCATTGTGCTGCCTGCTCCTTTTGGGCCTCCTGGGCTTCCTAGCTGCTCCCGGCGTCGCC   72
Pai Δ370     ATAAAGCTGATCGCATTGTGCTGCCTGCTCCTTTTGGGCCTCCTGGGCTTCCTAGCTGCTCCCGGCGTCGCC   72
Pai ΔSFHI    ATCAAGCTGATCGCATTGTGCTGCCTGCTCCTTTTGGGCCTCCTGGGCTTCCTAGCTGCTCCCGGCGTCGCC   72

Pai Dmel R6  TCGCCATCTCGCCACACTGGACCAGGAAACGGATCGGGATCTGGAGCTGGGTCCGGAAATCCGTTCAGGTCT  144
Pai Δ41      TCGCCATCTCGCCACACTGGACCAGGAAACGGATCGGGATCTGGAGCTGGGTCCGGAAA............  131
Pai Δ370     TCGCCATCTCGCCACACTGGACCAGGAAACGGATCGGGATCTGGAGCTGGGTCCGGAAATCCGTTCAGGTCT  144
Pai ΔSFHI    TCGCCATCTCG....................AATCGGGATCTGGAGCTGGGTCCGGAAATCCGTTCAGGTCT  124

Pai Dmel R6  CCAAGCTCACAGCAACGACCACTGTACTACGACGCTCCGATTGGGAAACCATCGAAGACTATGTACGCCTGA  216
Pai Δ41      ...............................................CCATCGAAGACTATGTACGCCTGA  155
Pai Δ370     CCAAGCTCACAGCAACGACCACTGTACTACGACGCTCCGATTGGGAAACCATCGAAGACTATGTACGCCTGA  216
Pai ΔSFHI    CCAAGCTCACAGCAACGACCACTGTACTACGACGCTCCGATTGGGAAACCATCGAAGACTATGTACGCCTGA  196
```

Figure S1. Nucleotide alignment of wild-type and mutant *Paillotin* alleles. Nucleotide sequences of wild-type *Paillotin* ($Pai^{Dmel\ R6}$) and the three mutant alleles ($Pai^{\triangle 41}$, $Pai^{\triangle 370}$, and $Pai^{\triangle SFHI}$) used in this study are aligned.

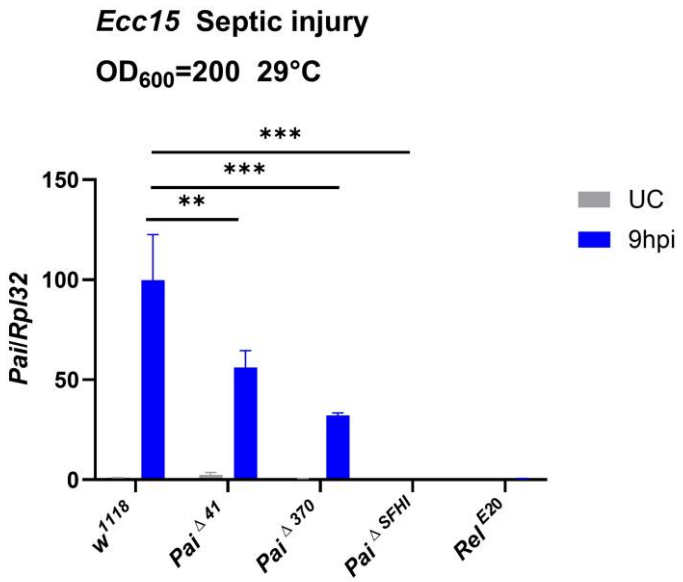

Figure S2: Expression of *Paillotin* in $Pai^{\Delta 41}$, $Pai^{\Delta 370}$, and $Pai^{\Delta SFHI}$ mutations. Expression was normalized to w^{1118} UC (unchallenged) flies set as a value of 1. The data shown in the figure are based on three independent experiments, with at least 30 flies per genotype at each time point in each experiment. Error bars represent SEM. Statistical analysis was performed using two-way ANOVA (** $p<0.01$, *** $p<0.001$).

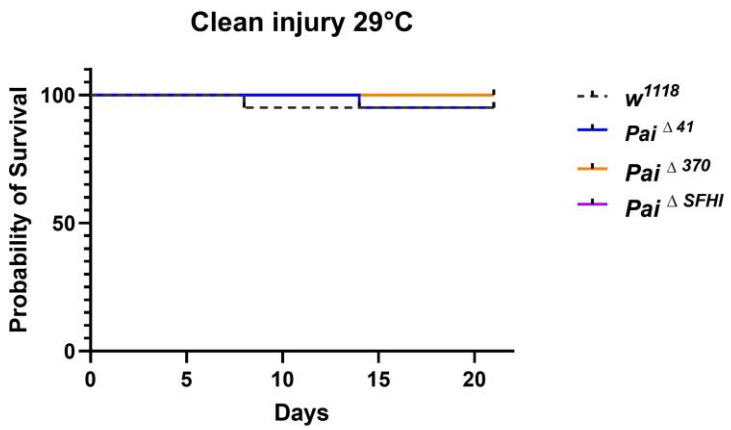

Figure S3: No susceptibility of *Paillotin* mutants to clean injury. The thorax of wild-type and mutants was pricked with a clean needle. A total of 80 flies were used in this experiment.

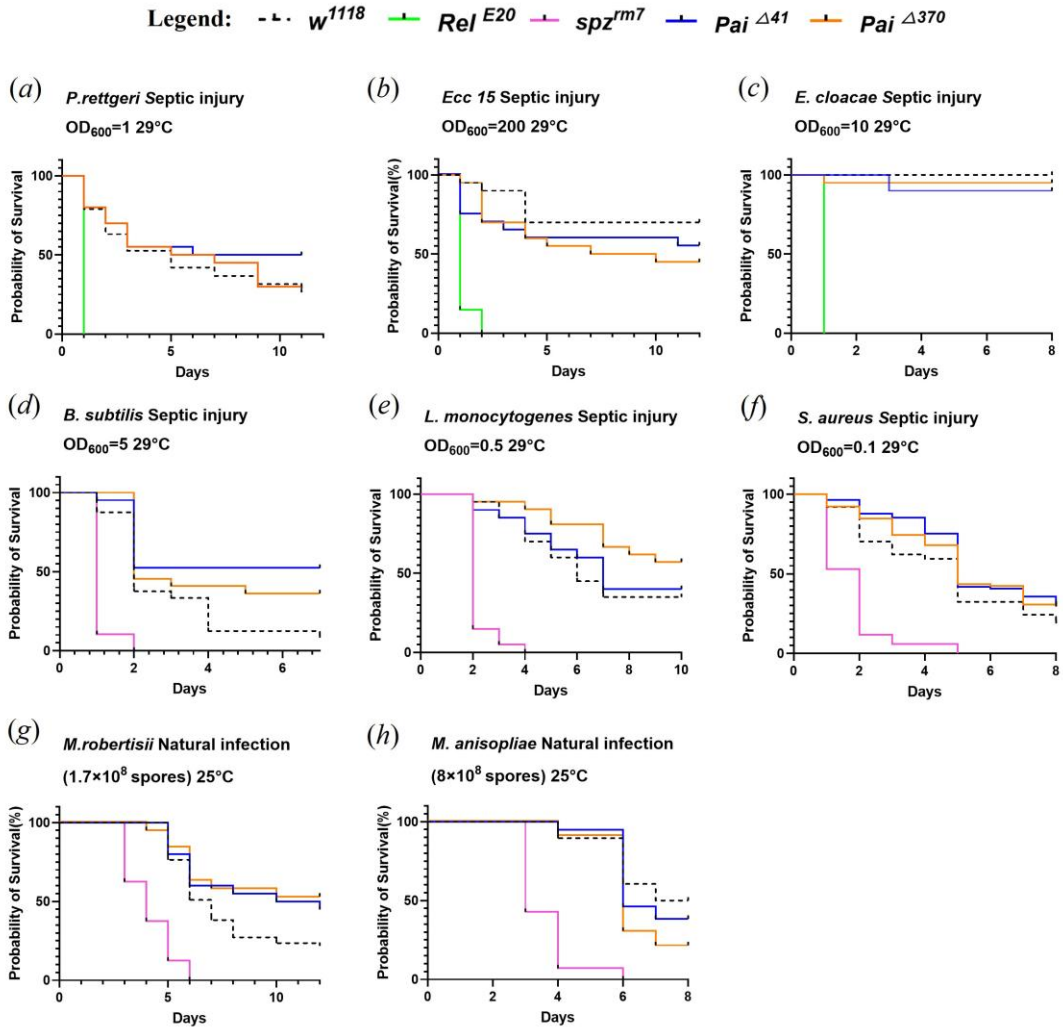

Figure S4: No consistent effect of *Paillotin* mutation on resistance to a broad spectrum of microbes. w^{1118} flies were utilized as wild-type controls, while spz^{rm7} flies lack the Toll pathway, and $Relish^{E20}$ flies lack the Imd pathway, were used as susceptible models for all survival experiments against infections. Tm and OD600 are indicated. Each survival assay was performed with a minimum of 80 flies.

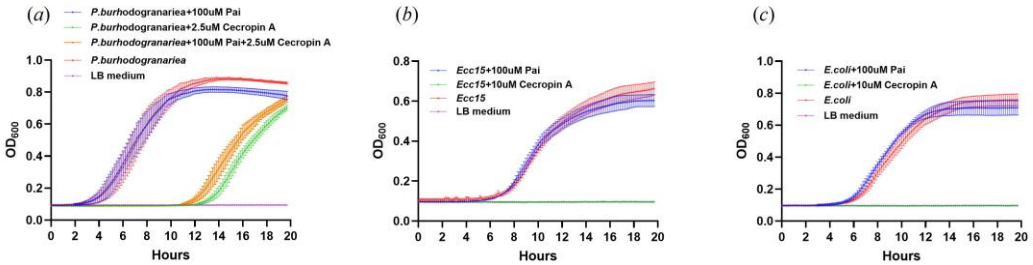

Figure S5: Paillotin did not show microbicidal activity *in vitro.* (*a*) The growth of *P. burhodogranariea* was monitored in the presence of the Paillotin peptide (100 µM) and/or the Cecropin A peptide (2.5 µM). The growth of *Ecc15* (*b*) and *E. coli* (*c*) was monitored in the presence of the Paillotin peptide (100 µM). Optical density (OD) values were measured every 10 minutes over 20 hours to generate bacterial growth curves. Different colored curves represent the conditions tested, as indicated in the figure legend.

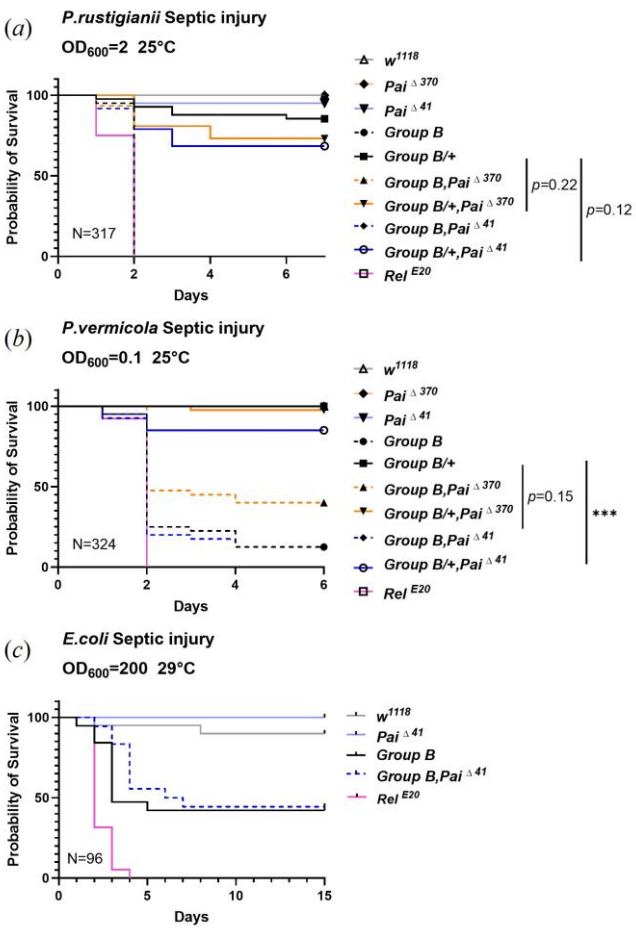

Figure S6: *Paillotin* shows a minor synergistic effect with *Group B* mutants against other *Providencia* species at the doses tested. (*a*) Both *Group B/+, Pai* compound mutants suffer a minor increase in mortality compared to *Group B/+* alone upon *Providencia rustigianii* infection. Although the synergistic effect between Paillotin and Group B was only marginally significant (Mutant, Cox Hazard Ratio (HR), *p*-value: *Paillotin*, HR =-0.01, *p*=0.904; *Group B*, HR =-2.8, *p*<0.001; *Paillotin*Group B*, HR = -0.41, *p*=0.09). (*b*) *Group B/+, Pai*$^{\Delta 41}$ mutants suffer a mortality increase compared to *Group B/+*. In addition, *Group B/+ Pai*$^{\Delta 370}$ shows no difference from *Group B/+*, but also *Group B, Pai*$^{\Delta 370}$ compound mutants survive better than *Group B* alone, indicating a complex phenotype after recombination for this infection that may mask the contribution of *Pai*$^{\Delta 370}$ at the chosen low-mortality dose (Mutant, Cox Hazard Ratio (HR), *p*-value: *Paillotin*, HR =0.15, *p*=0.14; *Group B*, HR =-2.7, *p*<0.001; *Paillotin*Group B*, HR = -1.13, *p*=0.03). Differences between *Pai*$^{\Delta 41}$ (truncated) and *Pai*$^{\Delta 370}$ (loss of start) may explain this difference. (*c*) No survival difference was observed for *Group B, Pai* compound mutants against *E. coli.* N=total number of flies in experiments.

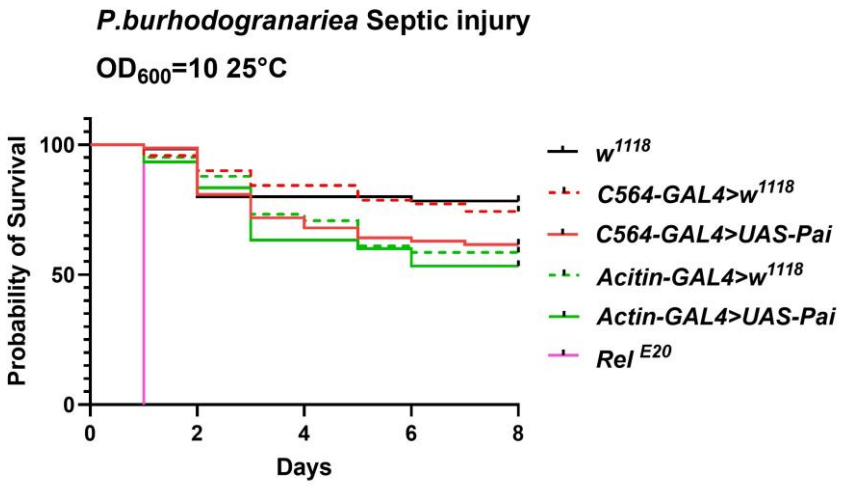

Figure S7: Overexpression of *Paillotin* does not confer protection against *P. burhodogranariea* infection in wild-type flies. Survival curves of flies overexpressing *Paillotin* by using *C564-Gal4* and *Actin-Gal4* drivers following septic infection with *P. burhodogranariea*. Survival assay was performed with a minimum of 120 flies.

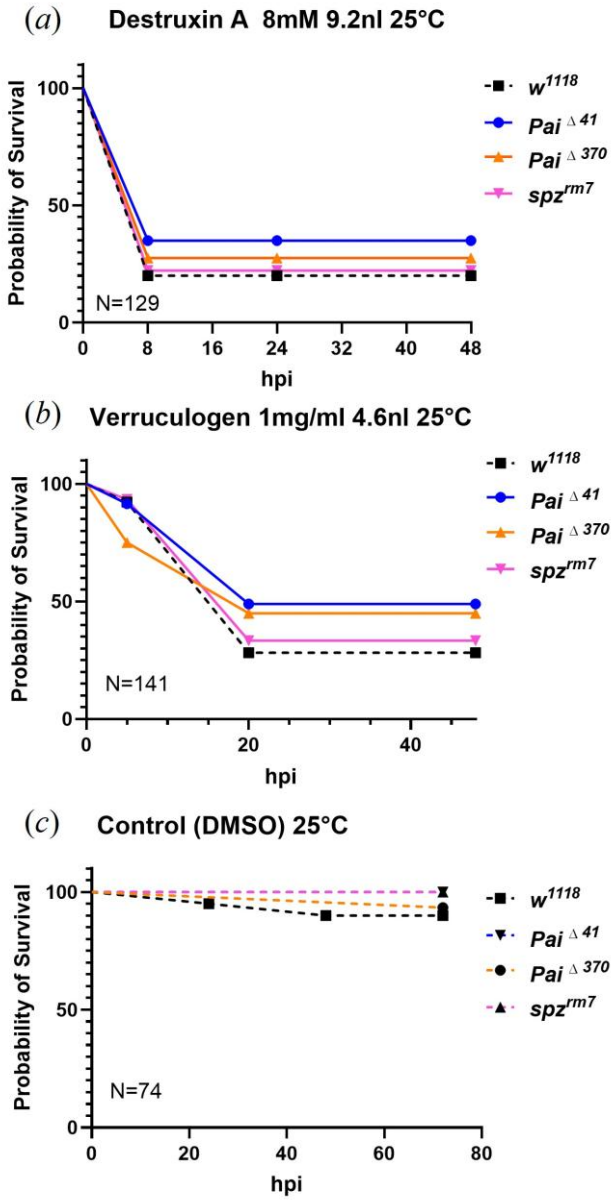

Figure S8: Paillotin does not contribute to protection against fungal toxins. (*a*) Survival of wild-type and *Paillotin* mutant flies following injection with 9.2 nl of 8 mM Destruxin A, a mycotoxin secreted by *M. robertsii*. (*b*) Survival of wild-type and *Paillotin* mutant flies injected with 4.6 nl of 1 mg/ml Verruculogen, a neurotoxin produced by *A. fumigatus*. (*c*) Survival of all tested fly strains following injection with DMSO as vehicle control. spz^{rm7} flies are used as positive controls. N=total number of flies in experiments.

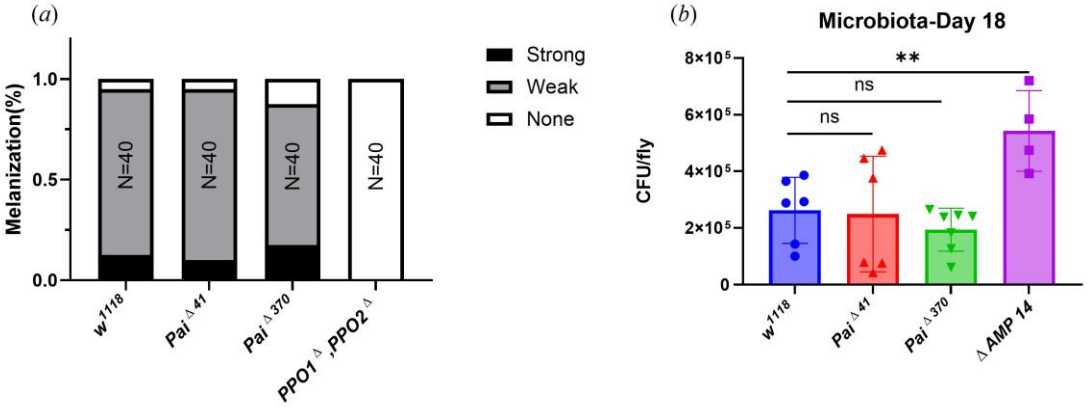

Figure S9: Paillotin is not involved in melanization response and microbiota regulation. (*a*) Cuticle blackening after clean injury is not impaired except in melanization-deficient flies (*PPO1*$^{\Delta}$*, PPO2*$^{\Delta}$). (*b*) CFUs per fly were measured for wild-type, *Paillotin* mutants, and the 14 AMP-deficient mutant (ΔAMP14). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test with w^{1118} as the control. A significant increase in bacterial load was observed only in ΔAMP14, while no significant differences were detected between wild-type and *Paillotin* mutants. Data points represent the mean bacterial load per fly, derived from individual experiments using pools of five flies per genotype (*** $p<0.001$, n.s. = not significant, $p>0.05$).

Table S2: Drosophila strains used in this study.

Drosophila lines	Reference
iso; iso; Rel^{E20}	Hedengrenetal.,1999
iso; iso; spz^{rm7}	Lemaitre et al.,1996
iso; Bom$^{\Delta 55}$; iso	Hanson et al., 2019
iso; iso; Rel^{E20}, spz^{rm7}	Ryckebusch et al., 2025
iso; Pai$^{\Delta 41}$; iso	DGRP
iso; Pai$^{\Delta 370}$; iso	DGRP
; Pai$^{\Delta SFHI}$;	Guangzhou Drosophila Resource Center
; ; UAS-Pai	This paper
iso ;Group B; iso	Hanson et al.,2019
iso ; PPO1$^{\Delta}$,PPO2$^{\Delta}$; iso	Bingelli et al., 2014
ΔAMP 14	Carboni et al., 2022
C564-Gal4	BDSC
Actin-Gal4	BDSC

Table S3: Microbial strains used in this study.

Type	Species	Strain,Source, DOI of previous study
Gram-negative	*Escherichia coli*	1106
	Pectobacterium carotovorum	Ecc15
	Providencia rettgeri	Dmel
	Enterobacter cloacae	B12
	Providencia burhodogranariea	B
	Providencia rustigianii	DSM 4541
	Providencia vermicola	DSM 17385
Gram-negative	*Micrococcus luteus*	doi:10.7554/eLife.44341
	Bacillus subtilis	168
	Enterococcus faecalis	254
	Listeria monocytogenes	BUG 1600
	Staphylococcus aureus	doi:10.7554/eLife.44341
Fungi	*Beauveria bassiana*	R444
	Metarhizium anisopliae	ARSEF-2575
	Metarhizium robertsii	ARSEF-2575